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Dating placentalia: Morphological clocks fail to close the molecular fossil gap

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Dating the origin of Placentalia has been a contentious issue for biologists and paleontologists. Although it is likely that crown-group placentals originated in the Late Cretaceous, nearly all molecular clock estimates point to a deeper Cretaceous origin. An approach with the potential to reconcile this discrepancy could be the application of a morphological clock. This would permit the direct incorporation of fossil data in node dating, and would break long internal branches of the tree, so leading to improved estimates of node ages. Here, we use a large morphological dataset and the tip-calibration approach of MrBayes. We find that the estimated date for the origin of crown mammals is much older, ~130–145 million years ago (Ma), than fossil and molecular clock data (~80–90 Ma). Our results suggest that tip calibration may result in estimated dates that are more ancient than those obtained from other sources of data. This can be partially overcome by constraining the ages of internal nodes on the tree; however, when this was applied to our dataset, the estimated dates were still substantially more ancient than expected. We recommend that results obtained using tip calibration, and possibly morphological dating more generally, should be treated with caution.

KEY WORDS: **Mammalia, molecular clock, morphological clock, placentalia, tip dating.**

Few subjects spark the interests of paleontologists, molecular biologists, and evolutionary biologists more than dating the origin of Placentalia. This was the classic example in the "rocks versus clocks," or "fossils versus molecules" debate (Benton 1999). Paleontological debates about the timing of the origin of crown Placentalia have been ongoing for the last three decades, with some phylogenies supporting a Late Cretaceous origin (Clemens 1970; Mckenna 1975; Szalay 1977; Archibald et al. 2011; Hooker and Russell 2012; Hooker 2014) and others a Cenozoic origin (Wible et al. 2007; Goswami et al. 2011; O'Leary et al. 2013). However, even the most conservative molecular estimates (dos Reis et al. 2012) date crown Placentalia and its earliest diversification deeper within the Cretaceous, and many studies have provided even older origin estimates (Bininda-Emonds et al. 2007; Meredith et al. 2011; Slater 2013). The earliest-known placental fossils require an origin of the clade at least in the latest Cretaceous (Signor and Lipps 1982), but this still leaves a large temporal gap between molecules and fossils (Foote et al. 1999; Archibald and Deutschman 2001). One controversial study has suggested that placental origination and diversification occurred exclusively in the Cenozoic (O'Leary et al. 2013), but this has been criticized for using hard minimum estimates and not considering maximum soft estimates to date nodes, as well as not accommodating preservation uncertainty (Springer et al. 2013; dos Reis et al. 2014). Efforts have been made to shorten the fuse between the molecular dating of Placentalia and its first fossil representatives (dos Reis et al. 2012). One possibility is to use fossils as tips in the phylogeny (Slater 2013), rather than purely in the direct estimation of internal node ages.

As nearly all fossils lack molecular data, the use of a morphological clock (Lewis 2001; Polly 2001) allows for the direct incorporation of fossils with morphological data (Pyron 2011) as tips in dating analyses. The morphological clock is now being employed in total-evidence analyses alongside molecular clocks (e.g., Ronquist et al. 2012; Wood et al. 2013; Puttick and Thomas 2015). Furthermore, the morphological clock has been used

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independently of the molecular clock to estimate clade ages (Schrago et al. 2013; Beck and Lee 2014; Lee et al. 2014). This is of particular promise to paleontologists, as clades without extant members can be used for clock analyses and for the estimation of the dated phylogenies that are essential in many macroevolutionary studies. In the case of the origin of Placentalia, tip calibration may also be able to break up long internal branches and in so doing improve estimation of the rates on early branches, and thus estimated dates (Magallón et al. 2010). In addition, tip calibration permits the more holistic inclusion of fossils in node dating, instead of treating fossil taxa only as minima for node calibration. This approach allows for the inclusion of more information from the fossil record to produce age estimates for nodes and so may help date the origin of Placentalia. Recently, Beck and Lee (2014) applied the morphological clock to Mammalia, and found a Late Jurassic/Early Cretaceous origin for Placentalia. This timing of divergence is much more ancient than predicted by either molecular clocks or the fossil record, and so perhaps represents an unrealistic estimate of that node's age.

Here, we use a large dataset of morphological characters, indeed one larger than any used in prior morphological clock analyses, to date the origin of the placental mammals, Placentalia. We use the morphological clock with both tip- and node-calibration approaches. Using a tip-calibration approach, we estimate an ancient, Early Cretaceous origin of Placentalia, in agreement with earlier morphological clock studies (Beck and Lee 2014), but contrasting with the much younger date estimated in a recent, definitive, large-scale molecular clock study (dos Reis et al. 2012). By contrast, a node-calibration approach yields age estimates that are much more congruent with dates estimated using a molecular clock. The ancient age estimates produced in this study may be due to the influence of tip dating or biases in the application of the morphological clock, or it may be a combination of these factors. Presently, it is not clear which of these factors is more important in driving ancient node age estimates. Consequently, it may be the case that the node ages obtained using morphological clock dating could be systematically overestimated in some or many studies in which this technique is employed (O'Reilly et al. 2015).

Materials and Methods

MODELING MORPHOLOGICAL EVOLUTION

To model morphological evolution on the tree, we used an equal rates, *k*-state, Markov (Mkv) model, with discrete gamma distributed rate heterogeneity among sites (Yang 1993; Lewis 2001). In the morphological model, a potential issue is that morphological data may include only variable characters, a problem known as acquisition bias, which leads to artificially extended branch lengths (Lewis 2001). Here, we correct this bias by using the Mkv model (Lewis 2001; Ronquist et al. 2012a).

DATA

We used the morphological dataset from O'Leary et al. (2013), which contains 4541 characters for 40 fossil and 46 extant taxa. We excluded characters with a low to zero chance of fossilization, such as behavioral and myological characters, resulting in a dataset pruned to include only the 3660 osteological characters. Furthermore, we reduced the number of taxa to 83 by removing the mammaliaforms *Morganucodon oehleri*, *Morganucodon watsoni*, and *Haldanodon exspectatus* to ensure compatibility between the root of the tree based on the O'Leary et al. (2013) dataset and that in dos Reis et al. (2012). All characters were treated as unordered as in the original O'Leary et al. (2013) study. In the analyses, we considered only variable, parsimony-informative characters (coding = informative, in MrBayes) when estimating topology and rate of evolution.

PHYLOGENETIC ANALYSIS

Analyses

We used MrBayes 3.2 (Ronquist et al. 2012) with a normal distribution for the clock rate prior and an exponential prior for the variance of the relaxed clock model with the relaxed clock Independent Gamma Rates (IGR) model (Lepage et al. 2007). We ran four chains on each of four runs for 20 million generations, sampling every 1000th generation. A conservative burn-in period of 25% of samples was discarded after the run had finished. We assessed convergence based on the estimated sample size (with a target value of above 200), potential scale reduction factor for each character (with a target value of below 1.01), and the standard deviations between chains (target below 0.05), as in Ronquist et al. (2012a). We also viewed all runs in Tracer (Rambaut 2014) to ensure stationarity was achieved.

Phylogenies

Overall, we performed three main analyses of the morphological clock with different modes of time calibration: (1) tip calibration on a phylogeny with no topological constraints, (2) tip calibration on a phylogeny constrained to the best solution in dos Reis et al. (2012), and (3) tip calibration alongside node calibration (Table 1).

We estimated two nonclock topologies to be used as fixed trees in the analyses. The two topologies were estimated in the following manner: for the first analysis we used a model with no topological constraints (unconstrained fixed tree; 1), and for the second we used a constrained topology to match the dos Reis et al. (2012) topology (constrained fixed tree; 2). These fixed trees are referred to as the unconstrained (1) and constrained (2) fixed trees, respectively.

The unconstrained fixed-tree topology (1) was virtually identical to the morphological analyses of O'Leary et al. (2013). This phylogeny is very different from that of dos Reis et al. (2012).

Table 1. Summary of the main analyses using the different phylogenies and dating methods.

Main phylogeny	Topology	Major analyses	Taxa included
(1) Unconstrained fixed tree	Nonclock phylogeny estimated with no topological constraints prior to dating analyses. Used as a fixed tree in dating analyses	Tip dating Tip dating (FBD model)	All species (148 Ma) All species (137 Ma)
(2) Constrained fixed tree	Nonclock phylogeny estimated with topological constraints to match dos Reis et al. (2012) prior to dating analyses; used as a fixed tree in dating analyses	Tip dating Tip dating (FBD model) Tip dating Tip dating	All species (146 Ma) All species (132 Ma) Extant species only (159 Ma) Extant Placentalia only (72.1 Ma)
(3) Constrained nonfixed tree	Phylogeny coestimated in the dating analyses using only the most complete characters (>60% completeness)	Tip dating Tip and node dating	All species (145.2 Ma) All species (110.8 Ma; see also Tables S2 and S3)

The ages following the taxa included refers to the estimated age of Placentalia. The numbering of the phylogenies is used throughout the text.

For example, the Atlantogenata (Afrotheria and Xenarthra) is not supported, and several clades that are found in molecular phylogenies are polyphyletic.

We constrained the extant species in the constrained fixed tree (2) to the same topology as in dos Reis et al. (2012), and we placed fossils in the same superorders as in O'Leary et al. (2013), but for the superordinal relationships we altered the topology to allow for a direct comparison to the dos Reis (2012) phylogeny. Therefore, fossil species were placed in the same superorders as indicated by the morphological and molecular analyses of O'Leary et al. (2013), but the topology of superorders and extant species were constrained to the topology of dos Reis et al. (2012). Unless stated, all analyses were performed on fixed trees (1 or 2).

Unless stated, all analyses were performed using a uniform tree prior. However, for the full datasets (1 and 2), we also used the fossilized birth–death process (Stadler 2010; Heath et al. 2014) in MrBayes. For the speciation parameter of the fossilized birth–death model, we set an exponential prior distribution (mean = 1), and for the extinction and sampling parameters, we set a prior beta distribution ($\alpha = 1, \beta = 1$).

Temporal constraints

For the calibration prior on the root age of the tree (age of crown Mammalia), we set a "soft bound" (Yang and Rannala 2006) by using an offset-exponential distribution with a minimum age of 124 Ma, and a 95th percentile age of 171.2 Ma—the same constraints used by dos Reis et al. (2012). However, dos Reis et al. (2012) used a Cauchy distribution, but this distribution is not an available option in MrBayes. Although this may mean ages are not directly comparable, it is unlikely to greatly influence interpretations. The Cauchy distribution is similar to the offset exponential in that it is heavy-tailed, and the expectation is that the fossil dates will be found close to the imposed hard minima (Inoue et al. 2010). The prior density on the root age for the morphological analysis was assigned as an offset exponential and uniform distribution, respectively, using the same dates as dos Reis et al. (2012) (min = 162.9, max = 191.1). Although dos Reis et al. (2012) used further dating constraints on internal nodes, the morphological dataset relied upon tip ages from the fossil species present in the phylogeny. The data were downloaded from *FossilWorks* (Alroy et al. 2014) on 27 February 2014 except for *Protolipterna ellipsodontoides* and *Dawsonolagus antiques* (O'Leary et al. 2013), and they were assigned uniform distributions.

Tip-calibration analyses

We performed analyses on the full dataset with exponential and uniform distributions on the root for the unconstrained (1) and constrained fixed trees (2). The temporal calibration information was from the tip dates, and the root distribution (offset-exponential or uniform).

Using the constrained fixed tree only (2), we also performed tip-calibrated divergence time analyses with phylogenies composed of extant taxa and extant placentals only. We changed the temporal constraints on the root—uniform and exponential with distributions identical to dos Reis et al. (2012)—to represent the origin of Placentalia when necessary.

As a test for the effects of character completeness on divergence time estimation, in the osteological only dataset, we systematically pruned characters according to their coverage across all taxa, and the analyses were repeated. We retained characters if they were present in a sufficient number of species; this threshold ran from 10 to 90% completeness, with analyses performed at 10% increments. We performed these analyses on the constrained fixed tree (2).

Node calibration

For the node-calibrated analyses, we applied node-calibrated divergence constraints on nontopologically fixed trees (3). It was only possible to achieve convergence when the columns of the data matrix had been subsampled to include only characters with 60% taxon coverage or higher. Therefore, we ran four node-calibrated analyses on datasets subsampled to contain characters with 60, 70, 80, and 90% or greater taxon coverage. We applied node-dating constraints to the following 13 internal nodes using an offset-exponential distribution: Theria, crown Marsupialia, Placentalia, Paenungulata, Xenarthra, Eulipotyphla, Cetartiodactyla, Carnivora, Chiroptera, Glires, Rodentia, Euarchonta, and Primates (see Fig. S1). When node dating was applied, we applied ages primarily taken from dos Reis et al. (2012), with supplemental dates from Benton et al. (2015) (see Table S1). As a direct comparison, using the same topological constraints, we also ran a tip-calibrated (no node age constraints) analysis on the trees after we removed characters that were coded for less than 60, 70, 80, and 90% of taxa.

EFFECTIVE TIME PRIOR

We ran analyses with no data (data = no) in MrBayes to obtain an estimate of the effective time prior that is informing the phylogenetic model. We plotted these alongside node age estimates from the posteriors in the model to gauge how much the data is influencing age estimates.

CLOCK-LIKE SIGNAL IN THE DATA

A correlation of Euclidean distance between species pairs in the cladistic matrix and time since divergence can give an indication of the predictive power of a dataset to act in a "clock-like" manner (Polly 2003a,2003b, 2004). Here, we use this test on the full dataset to test for evidence of "clock-like" behavior.

INFINITE SITES

As molecular sequence data approaches infinity, the confidence interval width surrounding posterior age estimates increases in a positive linear fashion with mean posterior age estimates. This is known as an infinite sites plot when applied to node-calibrated molecular phylogenies (Yang and Rannala 2006). We plot our data in the same manner to test how the addition of more data would influence analyses, and whether tip-dating approaches conform to the expected relationships as seen with molecular data and node calibrations.

FOSSIL DATING ALTERNATIVE: CAL3

Phylogeny dating methods can incorporate estimates of speciation, extinction, and fossil sampling rates, either independently of the morphological clock (e.g., Bapst 2013), or in conjunction with clock models (e.g., Heath et al. 2014). Methods have been developed to date phylogenies using occurrence dates of taxa, independently of a morphological clock model, by dating nodes according the occurrence of their first descendant (Hedman 2010; Bapst 2013; Brusatte et al. 2014). We used the cal3 function in the paleotree R package (Bapst 2012), which estimates ages using occurrence dates, but also samples node ages through use of a distribution that is a product of the speciation, extinction, and sampling rate for a clade. This method does not rely upon any models of morphological change, so can be used as a direct comparison to the morphological clock. Here, we used the function bin_cal3TimePaleoPhy to incorporate uncertainty in the age of tips on the phylogeny. The ages of tips were identical to those used in tip dating and used as first and last occurrence dates, with extant species set to 0 for both ages. We ran all analyses over 10,000 phylogenies, and used the constrained fixed topology from the morphological clock analyses as input. We used published estimates from the literature to find appropriate branching, extinction, and sampling priors in the model. Sampling estimates were taken from Foote et al. (1999), as 0.27–0.37 per lineage million years (Cenozoic) and 0.03–0.06 (Cretaceous), and from Tavaré et al. (2002) as 0.023 (Cenozoic) and 0.003 (Cretaceous). We took estimated speciation rates from Alroy (1999) and converted them to per capita rates per Ma (from per 2.5 Ma), giving a range of 0.18–0.604 and the mean gave an input value of 0.39. Similarly, we also used a value of 0.24 per capita extinctions per Ma from Alroy (1999). As the sampling values differ greatly between Foote et al. (1999) and Tavaré et al. (2002), we ran a second analysis using a sampling input of 0.013 per lineage million years, which is the mean from Tavaré et al. (2002).

COMPARISON OF MrBAYES AND BEAST2

To verify our results across alternative software, we used the morphological clock model in BEAST2 with an unconstrained topology in a tip-dating analysis with uniform age prior on the root. Furthermore, we used the Birth-Death Serial Sampling model (Stadler et al. 2013) tree prior that uses speciation rates, extinction rates, and sampling on sequentially sampled tips. This more complex model was used as a counterpoint to the simple uniform tree prior in MrBayes. In the BEAST2 analysis, we used the same priors for the speciation, extinction, and sampling rates as those we used in the cal3 analysis. We used the "BeastMaster" package in R (Matzke 2014) to produce the input files, and we ran analyses in BEAST2 version 2.1.3 (Bouckaert et al. 2014). The Markov chain Monte Carlo (MCMC) search consisted of four chains for 50 million generations sampling every 1000th generation, trace and tree files were combined, and we based convergence upon evolutionarily stable strategy (ESS) values and assessed convergence qualitatively by analyzing plots in Tracer.

Table 2. Morphological clock ages indicate an ancient origin for Placentalia.

	Full analysis		Extant-only taxa	Extant placentals
	FBD tree prior	Uniform tree prior	Uniform tree prior	Uniform tree prior
Mammalia	176.9 (162.9, 194.6)	167.3 (162.9, 177.3)	168.2 (162.9, 185.7)	NA
Marsupalia	46.6 (26.8, 70.1)	48.7 (27.5, 73.4)	50.9 (21.6, 83.1)	NA
Placentals	132.2 (119.1, 148.4)	146.2 (135.5, 156.8)	159.7 (141.4, 180.4)	72.1 (61.6, 106.5)
Boreoeutheria	130.3 (116.4, 145.4)	144.5 (133.2, 154.7)	156.2 (136.9, 176.9)	70.6 (58.2, 105.2)
Atlantogenata	127.7 (112.2, 143.5)	142.6 (129.8, 153.6)	155.1 (134.5, 176)	69.8 (55.7, 104.6)
Afrotheria	123.6 (108, 140.4)	138.5 (125.2, 150.9)	147.4 (124.6, 169.6)	66.7 (51.5, 100.3)
Xenarthra	66.1 (44.4, 92.1)	77.1 (51.2, 103.5)	97.5 (61.2, 133.5)	44.7 (25.4, 71.6)
Euarchontoglires	125.1 (109.8, 141.2)	139.1 (125, 151.3)	147.5 (124.1, 170.3)	67.9 (53, 101.8)
Laurasiatheria	128.3 (114.4, 143.1)	142.6 (131.2, 153)	153.3 (133.8, 174.3)	69.4 (56.3, 103.3)
Euarchonta	90.6 (77, 104.9)	99.2 (84.6, 114.4)	93.5 (64.6, 124.6)	44.3 (27.1, 69.2)
Glires	84.7 (66.3, 103.8)	96 (75.1, 117.9)	102.5 (74.4, 132.3)	46.4 (30.7, 71.2)
Eulipotyphla	91.2 (68.4, 114.7)	106 (80.2, 129.4)	115.6 (84.6, 146.4)	51.6 (33.5, 78.7)
Carnivora	33.4 (17, 50.2)	34.4 (17.5, 53.8)	38.2 (15.2, 64.8)	17.5 (5.7, 32.1)
Certartiodactyla	85.6 (72.7, 100.4)	98.9 (83.5, 114.3)	104.3 (77.8, 132.9)	47.9 (32.1, 73.9)
Chiroptera	80 (65.8, 96.1)	88 (71.2, 106.9)	77.7 (49, 108.8)	35 (20.2, 56.1)
Rodentia	53.4 (38.2, 67.7)	56.4 (39.7, 72.8)	69 (45.2, 98.2)	32.2 (17.8, 51.6)
Paenungulata	101.6 (84.9, 119.5)	115.3 (97.2, 133.1)	112.3 (82.2, 141.2)	52.7 (34.5, 80.7)
Cow/horse	122.8 (108.9, 137.2)	137.4 (125.7, 149.4)	142.4 (119.2, 164.9)	64.8 (50.2, 96.8)
Pig/cow	82.7 (69.2, 96.8)	95.8 (80.8, 111.9)	97.4 (71.4, 125.9)	45.2 (29.5, 70.1)
Dolphin/cow	80.7 (67.1, 94.7)	93.5 (78.2, 109.1)	91.5 (65.2, 119.4)	42.9 (27.4, 66.7)
Horse/cat/bat	119.7 (105.1, 134.1)	134.3 (121.6, 146.1)	137.9 (114.2, 161)	62.8 (48.2, 94.1)
Horse/cat	117 (102.6, 131.8)	131.5 (118.3, 144)	132.9 (108.2, 157.2)	60.8 (45.7, 91.7)
Human/tarsier	42.7 (29, 57.9)	46.1 (28.7, 64)	52.7 (30.8, 77.2)	24.3 (13.1, 40.3)

When both the fossilized birth–death process (FBD) and uniform root prior distributions are employed for the full tree, an ancient date is produced spanning the Late Jurassic to Early Cretaceous. Similar results are found with just extant taxa and just extant Placentalia.

Results

TIP CALIBRATION

Our tip-dating analysis with the morphological clock indicates a Late Jurassic to Early Cretaceous origin for Placentalia. The osteological dataset of 3660 characters with the constrained fixed tree (2) gives an origin of 146.2 Ma (135.5–156.8, 95% highest posterior density [HPD]) for Placentalia with the uniform tree prior, and 132.2 Ma (119.1–148.4, 95% HPD) with the fossilized birth–death tree prior (Table 2, Fig. 1). The majority of comparable nodes are older than given by dos Reis et al. (2012), with a few exceptions, including the root (Fig. 2). Results are very similar when the tree age prior is set to a uniform distribution (Table S2).

Similar results are found for the age of Placentalia when the unconstrained fixed phylogeny (1) is employed: a Late Jurassic, or Early Cretaceous age (148∼150 Ma) is seen with both the exponential and uniform prior on the root (Table 3).

Dates from the analyses are generally much older than the molecular estimates of dos Reis et al. (2012). Node-by-node comparison indicates that nearly all nodes, with the exception of the root, are older in our morphological clock study than using the molecular clock (Fig. 2c). Similarly, our node ages (Fig. 2c) are broadly comparable to the morphological clock analyses of Beck and Lee (2014), but the ages in our study tend to be slightly younger. Additionally, the Placentalia age found by Beck and Lee (2014) is older (163.7 Ma) compared to the median age (146.4 Ma) in our analysis. However, when we used the fossilized birth–death tree prior the estimated ages were lesser (Fig. 2d) than those found by Beck and Lee (2014).

Inclusion or exclusion of all extant taxa does not make a great difference to estimated dates for the origin of Placentalia. This is seen with the use of an exponential (159.7 Ma) and uniform (166.6 Ma) prior (Table 2 and Fig. 3). A younger Late Cretaceous age of 72.1 Ma arises when dating only extant Placentalia (Fig. 3), but the uncertainty stretches back from the Paleogene to the mid Cretaceous (61.6–106.5 Ma).

Reducing the size of the dataset to include only the more complete characters shows there is little trend related to dataset size (3). The root age ranges from 165.2 to 171.9 Ma and the estimated age of Placentalia from 145.2 to 165.3 Ma (see Table S3).

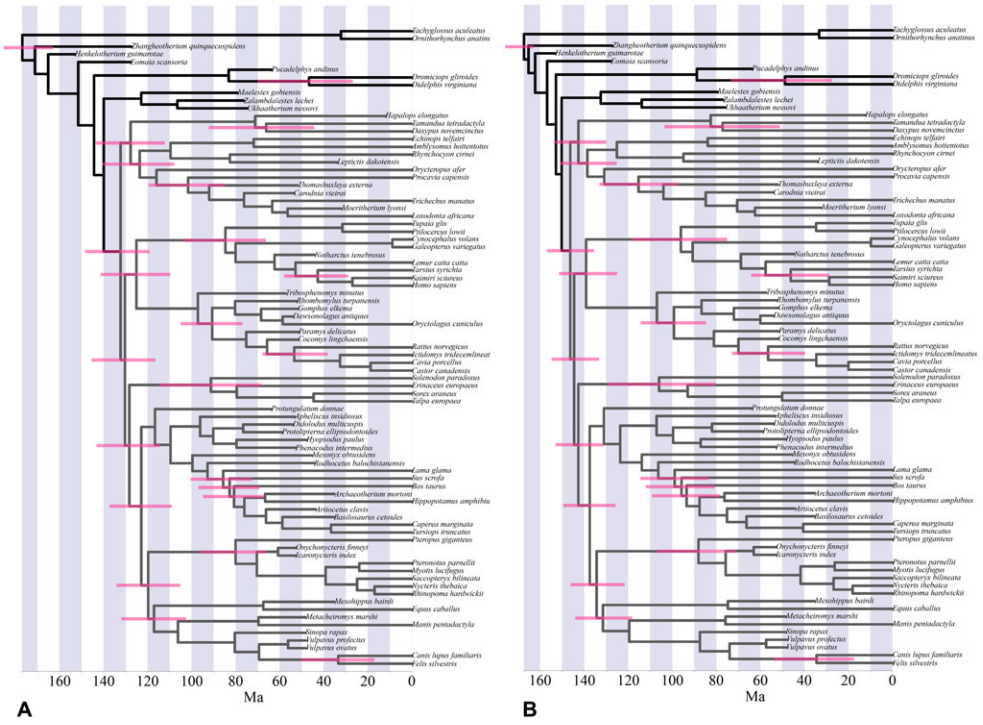

Figure 1. **Time-calibrated Mammalia phylogeny indicates that Placentalia originated around 145 Ma with the uniform tree prior (A) and around 130 Ma with the fossilized birth–death tree prior (B). Most cladogenesis occurred prior to the Cenozoic, when using the full dataset and fixed tree. The pink bars indicate the 95% confidence intervals (CIs) around the node age estimates for the nodes that are directly comparable to dos Reis et al. (2012).**

NODE CALIBRATION

In comparison to tip dating alone, slightly younger ages are found when internal node dating is used alongside tip dating (Table 4 and Fig. 3). As discussed (see Methods), it was difficult to achieve convergence with datasets unless we excluded characters with less than 60% taxon coverage. Using this dataset, and smaller datasets (3) with fewer characters, results in age estimates of Placentalia (~108–120 Ma) that are younger than those from comparable tip-dating analyses (Table 4; Tables S3 and S4). However, the age estimates for Placentalia and other nodes are still more ancient than molecular estimates.

BRANCH RATES THROUGH TIME

Early branches in the tree have higher median rates when using the full dataset on the constrained and unconstrained phylogenies (Fig. S2); this effect is slightly lessened in the reduced tree dataset, and disappears when internal node calibrations are used alongside tip dating (Fig. S2).

PRIOR AND POSTERIORS

Given different time priors, the ages of clades converge on the same values for posterior age estimates (Fig. 4). The effective prior differs between the uniform and fossilized birth–death priors in analyses, but the posteriors tend to converge on similar ages.

CLOCK-LIKE SIGNAL

Evidence for clock-like behavior is weak, but not entirely absent. Although there is evidence of a curvilinear relationship (species with older common ancestry are more dissimilar), it is not significant (Mantel–Spearman test, r statistic $= 0.07$, $P = 0.197$; Fig. 5).

INFINITE SITES PLOT

We find that in our analyses, there is not a perfect linear relationship between posterior age means estimates and confidence intervals (Fig. 6). For all analyses there is a lower range of HPD surrounding nodes closer to the root, than younger nodes that are opposite to the expected relationship (Yang and Rannala 2006). The range of R^2 (~0.7, when forced through the origin) values indicates that the addition of more data may improve estimates, but the distribution indicates that it is unlikely to occur in the expected linear manner.

cal3

We found a median age of 67.2 Ma for Placentalia (64.8, 73.6 Ma) when mean estimates of sampling rates were applied alongside speciation and extinction rates (Fig. S3). Even when the lowest estimated sampling rate was employed (0.013), the median age (75.9) and upper confidence interval, 94.4 million years (Myr)

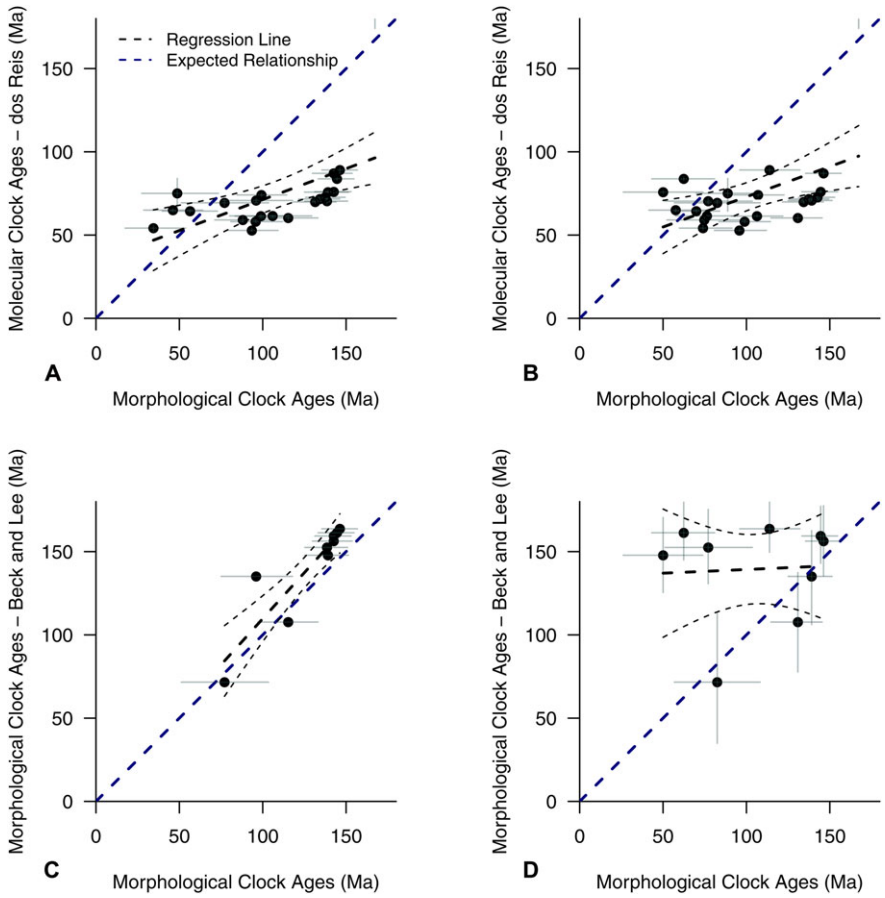

Figure 2. **Comparison of the morphological clock ages with the molecular estimates of dos Reis et al. (2012) indicates that many morphological estimated nodes are older than molecular clock estimates (A, B), but are very similar to another morphological clock study of Beck and Lee (2014) (B, C). Ages are slightly older when using the uniform tree prior (A, C) compared to the fossilized birth–death tree prior. Furthermore, the morphological clock estimates from this study have large confidence intervals (gray lines). The regression line (dotted black) with 95% CIs is much shallower than the 1:1 expectation (dotted gray) than the molecular estimates (A), but overlaps with the other morphological clock study (B).**

Table 3. **Topological constraints have little effect on the age estimates for Placentalia.**

	Root	Placentals	Marsupials
Dos Reis	185.0 (174.5, 191.8)	89.9 (88.3, 91.6)	66.7 (50.7, 83.7)
Uniform tree prior	169.0 (62.9, 181.8)	148.7 (137.4, 162.9)	94.6 (76.4, 115.7)
FBD tree prior	182.5 (164.9, 204.4)	137.4 (121.8, 152.9)	73.4 (64.4, 85.9)

Using the unconstrained topology (1) produces ancient ages compared to the estimates of dos Reis et al. (2012) when using a uniform tree prior and the fossilized birth–death tree prior.

were younger than any of the morphological dates estimated here (Fig. S3).

COMPARISON OF MrBAYES AND BEAST2

Analyses in BEAST2 produced ages and an identical topology to those obtained in MrBayes. The median age for Placentalia from the BEAST2 model (136.4 Ma) is million years (Myr) younger than the comparable age from MrBayes (137.4 Ma). The confidence intervals around estimated ages were similar, but the upper and lower posterior densities from MrBayes (121.8, 152.9) were smaller than those from BEAST2 (117.3, 158.1).

Discussion

The ages for deep nodes in the phylogeny of Mammalia presented here do not close the gap between the fossil record and molecular estimates of the age of origin of Placentalia. In fact, only one age from the various morphological clock analyses is younger than

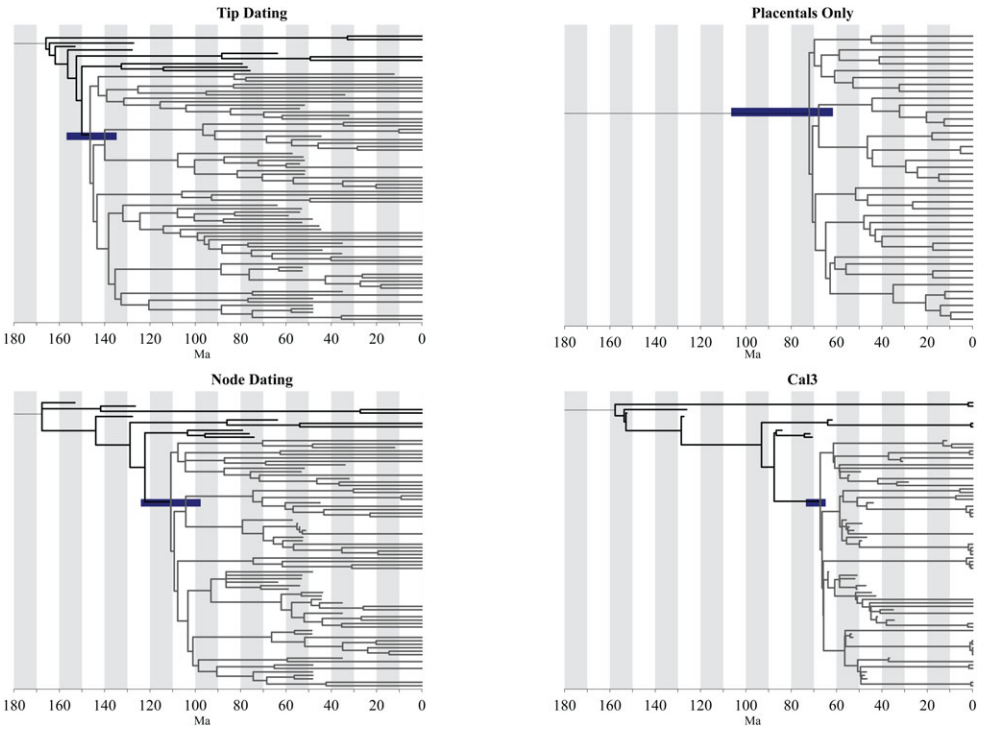

Figure 3. Different dating methods produce very different origin estimates for Placentalia (gray), and differ in their uncertainty (blue bars represent 95% CIs). The morphological clock points to a divergence well within the Mesozoic when tip dating (Tip Dating), or node and tip dating (Node Dating) is used. The morphological clock does indicate an Early Cretaceous origin if only extant Placentalia (Placentals Only) are employed in the analyses, but these dates may rely upon prior assumptions. Using a nonclock model (cal3) gives a late Mesozoic to early Cenozoic origin for Placentalia.

most molecular clock estimates, and this is only when extant Placentalia are used in analyses. The ancient date estimates obtained in this study cannot be attributed to a failure of the fitted models to account sufficiently for fossil sampling, as ancient dates are still obtained when we analyzed data from only extant taxa. Additionally, posterior age estimates differ from prior age distributions, which indicate that the priors were not having an overly large effect on age estimates. The ancient ages produced here are due in some part to a lack of internal node constraints forcing ancient ages (Arcila et al. 2015), but there also seem to be some inherent factors in the morphological dating method that produce ancient ages (O'Reilly et al. 2015).

Many of the ages presented here are older than molecular clock estimates, and what is known from the fossil record. As with Beck and Lee (2014), morphological clock models have shown the age of Placentalia to be Early Cretaceous (around 145 Ma). Other evidence suggests that the origin of crown Placentalia actually occurred in the Late Cretaceous. The fossil record of the Late Cretaceous is well studied, and a number of nonplacentals (Ji et al. 2006; Wilson et al. 2012) and placental mammals (Archibald et al. 2011; Hooker and Russell 2012; Hooker 2014) are known. Close study has resulted in substantial revisions of the fossil materials (see Wible et al. 2007; Goswami et al. 2011; O'Leary et al. 2013), and the fossils of crown Placentalia just are not there, despite the availability of suitable locations with comparable fossil specimens scattered around the world. Therefore, fossil evidence (Benton 1999, 2015), and direct analysis of preservation probabilities on fossil distributions (Signor and Lipps 1982; Foote et al. 1999; Archibald and Deutschman 2001) indicate that a "long fuse" stretching back into the Cretaceous is unlikely for Placentalia. Although it is possible that the record is genuinely poor, or that some Cretaceous mammals really are placentals, but lack apomorphies (Foote et al. 1999), there is little direct evidence to support a deep Cretaceous origin for Placentalia. Thus, the main date estimates from this study appear to be unrealistic both when compared to a close reading of the fossil record (with preservation probability taken into account), and when compared to the molecular clock estimates obtained in other research.

In tip calibration, the absence of many internal prior time constraints allows for age estimates to be pushed toward the root (Arcila et al. 2015). In molecular dating with internal constraints, it has been shown that confidence intervals in posterior age estimates increase in a linear fashion with distance from the root as measured by mean posterior age estimates (Yang and Rannala 2006). Here, for two tip-calibration approaches (Fig. 6), the HPD does not follow this expected linear relationship, and smaller

Table 4. Node-dating constraints plus tip dating bring estimated dates that are closer to the fossil record compared with tip dating alone.

	Tip dating	Node-dating constraints
Mammalia	165.2 (162.9, 173.1)	167.6 (162.9, 179.7)
Marsupials	51.9 (23.4, 81.1)	53.9 (48.6, 67.3)
Placentals	145.2 (125.9, 163.4)	110.8 (97.6, 124)
Boreoeutheria	142.5 (123.2, 160.9)	109.3 (96.1, 122.5)
Atlantogenata	140.3 (119.7, 159.5)	107.7 (93.6, 120.8)
Afrotheria	111 (81.8, 138.5)	104.4 (90.2, 118.4)
Xenarthra	91 (59.6, 124.8)	70.3 (55.6, 88)
Euarchontoglires	134.9 (112.1, 154.3)	104.2 (89.9, 118)
Laurasiatheria	139.9 (120.5, 158.3)	107.8 (93.8, 120.3)
Euarchonta	84.7 (66.9, 105.9)	74.6 (61.9, 88.3)
Eulipotyphla	95.1 (69.2, 123.5)	74.5 (61.5, 92.8)
Carnivora	97.2 (63.9, 130.7)	42.3 (39.7, 49.9)
Certartiodactyla	34.9 (12, 59.1)	44 (30, 58.7)
Chiroptera	55.5 (31.6, 81.5)	51.7 (48.7, 59.8)
Rodentia	66.2 (45.6, 88.7)	56.6 (55.6, 59.7)
Paenungulata	52.2 (34.7, 69.3)	71.7 (55.6, 87.7)
Cow/horse	87 (57.9, 118.7)	103.3 (89.5, 115.9)
Pig/cow	132 (112, 152)	44 (30, 58.7)
Dolphin/cow	55.5 (31.6, 81.5)	57.5 (52.4, 69.9)
Horse/cat/bat	72.9 (43.2, 102.2)	101.1 (87.7, 113.9)
Horse/cat	128.6 (108.2, 147.7)	98.6 (85.1, 111.6)
Human/tarsier	125.2 (105.8, 145.7)	43.3 (28.8, 55.9)

When using the nonfixed, constrained tree (with the reduced dataset), a similar age to the fixed tree is observed with tip dating only, but these ages are generally younger in the analysis that also incorporates node constraints.

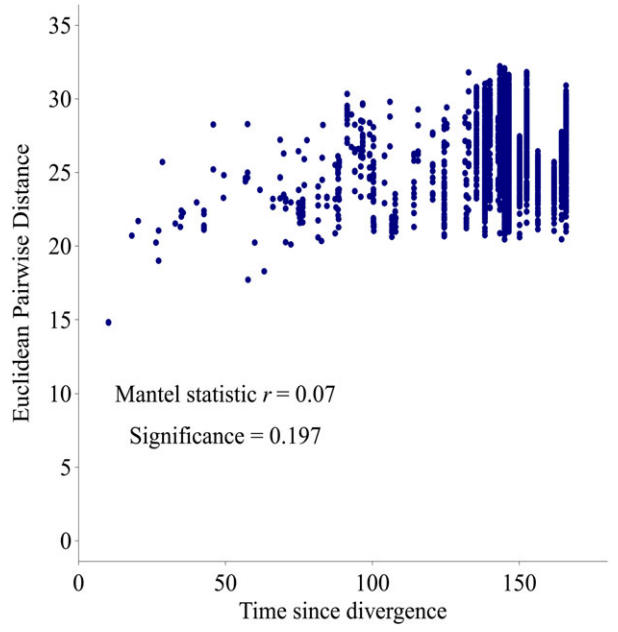

Figure 4. There is a nonsignificant relationship (Mantel–Spearman rank test, $P = 0.197$) between time since divergence and generalized Euclidean distance separating sister species at the tip of a phylogeny.

confidence intervals in posterior ages are found closer to the root. This phenomenon is evident with both the use of the uniform and fossilized birth–death tree priors, but is more pronounced with the uniform tree prior. This pattern even appears when node constraints are applied—indeed there is a close correspondence between the plot for the fossilized birth–death tree model and node calibrations under the uniform tree prior. The use of dates from tip-dating analyses in an infinite sites plot may be considered to be unjustified, as the theory of these plots is based upon nodes with time calibration, and they are used to indicate the limits of precision when infinite sequence data are analyzed (Yang and Rannala 2006). Here, we are using tip calibration and morphological data, and we use these plots to show that the assumption of an infinite sites plot may not hold true with tip calibration. The root constraint may provide an upper bound, and so shorten the upper (older) confidence intervals. In contrast, and unlike node dating analyses, internal nodes are not constrained, so the expected positive relationship between node age and uncertainty may not be applicable (Fig. 6). Our results indicate that in tip calibration there is a bias toward ancient ages (O'Reilly et al. 2015) and this problem may be partially overcome by applying internal node calibrations (Fig. 3). We only applied eight node

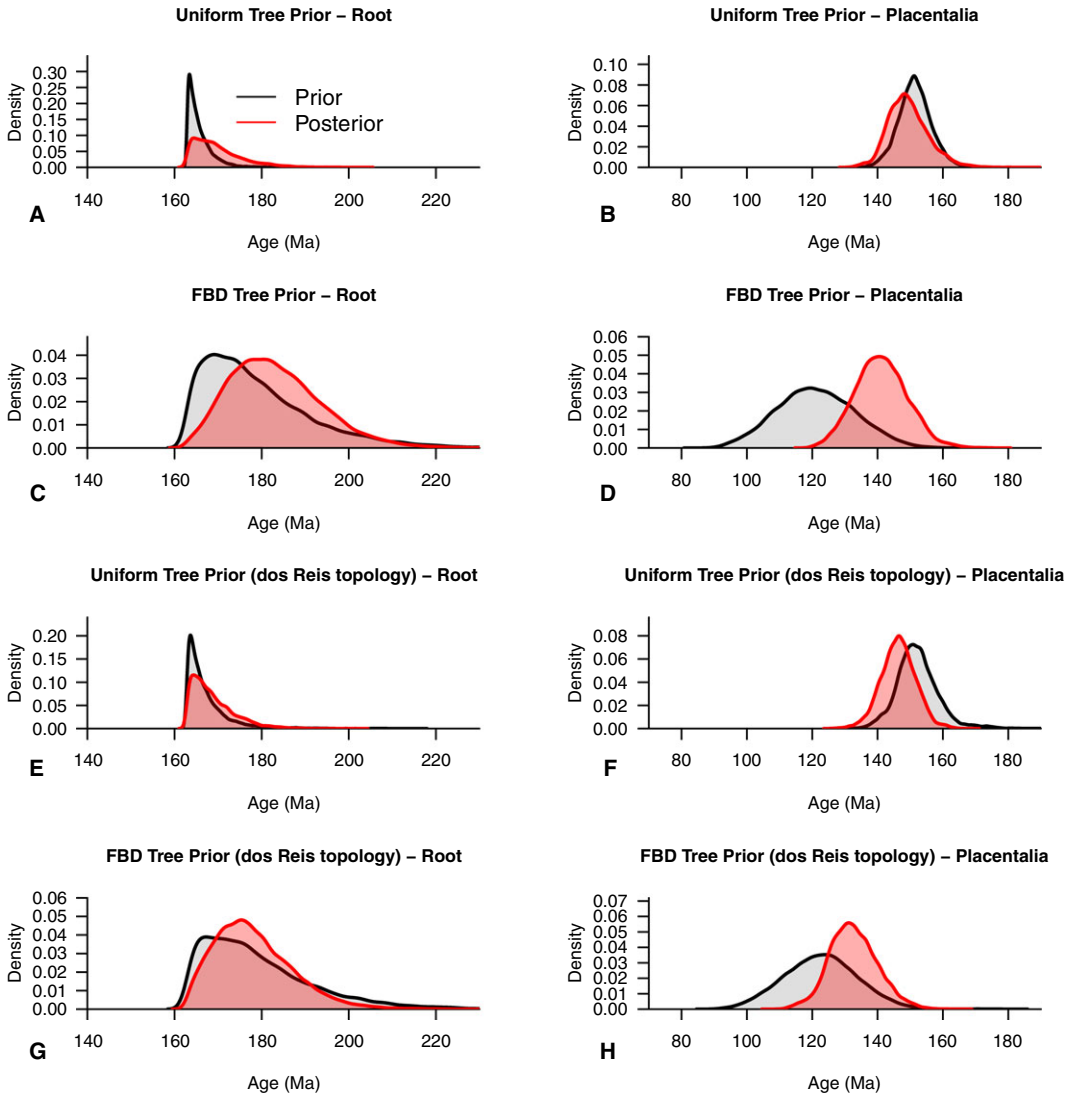

Figure 5. **Comparisons of effective priors and posteriors for the root node and Placentalia indicated the impact of data on analyses. With the unconstrained fixed tree (A–D) and constrained fixed-tree (E–H) similar patterns are shown for the uniform tree prior—a tendency to move to a younger age (A, B, E, F). For the fossilized birth–death prior (C, D, G, H) there is a move to a more ancient age.**

calibrations, so perhaps more node constraints would produce ages more congruent with molecular divergence estimates. However, even with node constraints (Fig. 3) or extant-only analyses, dates are always older than expected. This could be due to the evidence we have of weak "clock-like" signal in the data, or it is also possible that morphological dating models simply tend to give ancient age estimates (O'Reilly et al. 2015).

The assumption of a clock-like, relaxed model of morphological evolution, similar to that of the molecular clock (Zuckerkandl and Pauling 1962), has not been thoroughly tested (O'Reilly et al. 2015). Additionally, while rates and assumptions of clock-like evolution can vary with molecular data, the coding of molecular characters is consistent across studies and datasets. Morphological coding can vary infinitely, comprising both continuous and discrete characters, characters of different magnitude, different intensity of coding of different anatomical regions, and characters that are subdivided to a greater or lesser extent—these factors are likely to influence the ability to infer clock-like behavior for morphological characters. Further, there is evidence for clock-like behavior in morphological traits in mammal teeth (Polly 2001), but the way in which cladistic character sets are constructed means differences between taxa increase with taxonomic scope, but not necessarily with absolute time (O'Reilly et al. 2015; D. Polly, pers. comm.). The equivocal evidence of a clock-like signal shows that, although there is some time-related signal in the data, it is not significant (Fig. 5).

A potential problem here could be specific problems with clock models in MrBayes: very ancient dates have been obtained

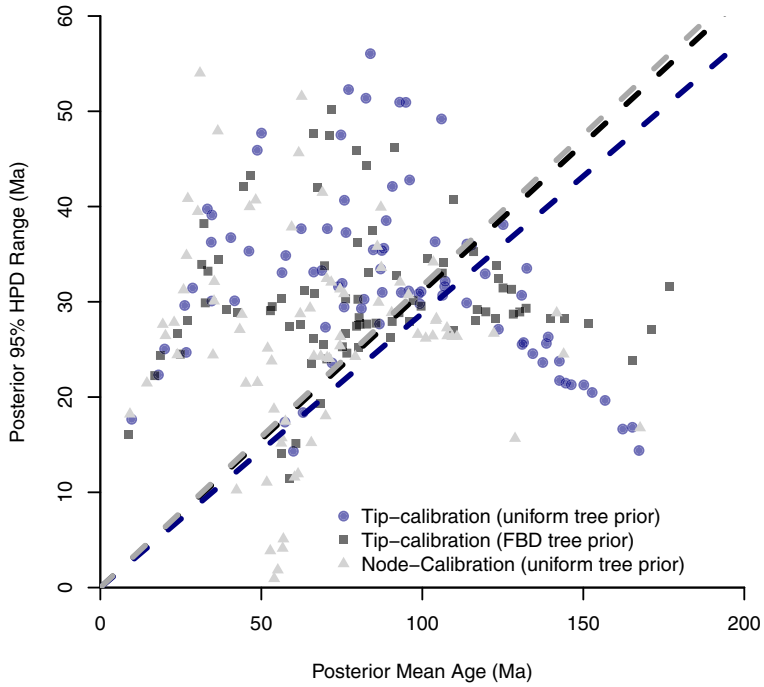

Figure 6. **Infinite sites plot of tip calibration (uniform and Fossilized birth-death process FBD tree prior) and node calibration indicates that the lack of internal time constraints on nodes means ages are pushed back so highest posterior density (HPD) range of nodes is smaller and closer to the root due to the effect of the root constraint. This effect is most evident with the uniform tree prior and lessened by the FBD tree prior and internal time calibrations.**

in clock models (Beck and Lee 2014), and studies have found clock artifacts (Lee et al. 2013) in MrBayes. On the other hand, studies that have shown the greatest congruence between molecular and morphological dates have employed alternative software, such as Beast (Drummond et al. 2012; Schrago et al. 2013). In our MrBayes tip-dating analyses, we observed some surprising results, for example, using more data made it more difficult to achieve convergence, and an unexpected spike in rates of morphological evolution occurs near the root (Fig. S3). Some of these problems have been reported before (Lloyd et al. 2012; Beck and Lee 2014). The spike in rates near the base may be due to divergence of major clades, and it has been suggested that these high rates can influence divergence times (Beck and Lee 2014). This spike does not appear attributable to fossils near the base, as it alters when using different time constraints even when identical fossils are included (Fig. S2). To check that we were not reporting results unique to a particular software implementation, we repeated all analyses with BEAST2 (Bouckaert et al. 2014), and found the same ancient ages as in MrBayes. Additionally, factors such as increasing character conflict as datasets increase in size may explain difficulties in achieving convergence with larger character matrices. Therefore, we do not believe that these reflect specific software idiosyncrasies or bugs.

In the O'Leary dataset, fossil sampling is not evenly distributed through time, and is mainly concentrated around the K-Pg boundary. In the future, more evenly distributed sampling, or models that incorporate uneven sampling (Drummond et al. 2012; Zhang et al. 2015), will be necessary to accurately model tree priors and divergence times. Therefore, the current tree priors may not accurately capture variations in sampling and so may be a problem for the dating analyses, and could potentially be improved to reflect variable sampling through time. However, the fact that there are problems with dating in models with no fossils (Tables 1 and 2) suggests that it is unlikely that variable sampling is the key or sole explanation of problems with morphological clock models.

Though some prior studies have found congruence between morphological and molecular clock dating within mammals (Schrago et al. 2013), at our larger taxonomic scale there seems to be little evidence of agreement between these two sources of data on node ages (Meredith et al. 2011; dos Reis et al. 2012, but see Bininda-Emonds et al. 2007; Fig. 2). One issue may be taxonomic breadth; Schrago et al. (2013) analyzed only New World primates, rather than all Mammalia, although they also used estimates from molecular divergence times to calibrate age priors. It may be that smaller clades show more uniform rates of morphological evolution across all branches, whereas among Placentalia as a whole, there are substantially different rates of morphological change within different subclades, or at different times. Some of these issues in improving the model fit

for morphological clocks could possibly be resolved by using lineage-specific rates (Beaulieu et al. 2013) and a priori testing for "clock-like" behavior of characters (Clarke and Middleton 2008; Jarvis et al. 2014). Different methods, such as node-dating constraints alongside tip dating, may be necessary to bring congruence to estimates (Table 3 and Fig. 3).

Effective priors in relaxed clock analyses do not always follow the user-set time priors (Warnock et al. 2012, 2015), and when analyzing divergence times, careful selection and judgment of time priors is essential (Warnock et al. 2015). Here in the tip-dating analyses, as no node-based priors (with the exception of the root) are specified, it is unclear how tip-based time priors will influence the posterior; these node estimates will come from the joint priors of the tree model and of the tip times. Effective priors on the nodes appear to be quite old (Fig. 4), and seem to influence the posterior estimates. Currently, more data does not improve the situation, so it may be important to produce models that provide sensible priors (Warnock et al. 2012) rather than adding more character data. The effective priors differ between the uniform tree prior and fossilized birth–death tree prior analyses, but the data still produce ancient ages in the posterior (Fig. 5). Yet, another solution is to use morphometric data that have been shown to act in a clock-like manner. For example, tooth characters in some mammals have been shown to change in a linear fashion with time (Polly 2001; Gomez-Robles et al. 2013), but reach saturation rapidly, meaning the ability to estimate divergence times is lost (Polly 2001). Therefore, morphometric data from dentition of basal Placentalia (Late Cretaceous–Early Paleogene) could be used, as the data would have not yet reached saturation and would provide the appropriate temporal resolution (D. Polly, pers. comm.).

When sampling and diversification rates are considered alongside occurrence dates, independently of the morphological clock, the dates produced are more congruent with the fossil record. Here, we employ a method that incorporates branching, extinction, and sampling estimates (Bapst 2013) alone (independent of the morphological clock or character change) to act as time priors on nodes and to coalesce on a time of common ancestry for Placentalia. This method can increase the congruence between the fossil record and molecular clocks (Fig. 3 and Fig. S3). Clearly, these estimates are sensitive to prior assumptions (Fig. S3), as the lower sampling rate input gives an older age estimate, mainly due to the greater uncertainty in the model. Thus, there is reason to favor the use of the higher sampling rate as it is applicable to all Mammalia (Foote et al. 1999) rather than just primates (Tavaré et al. 2002), as was the case for the lower sampling rate. However, this argument may become circular: if we assume a low sampling rate, then we can more or less accept molecular clock estimates, and therefore a lower sampling rate makes sense. Yet, even this low sampling rate provides an age that is much younger than the morphological clock estimates, and most molecular estimates too (Bininda-Emonds et al. 2007; Meredith et al. 2011).

Conclusions

The morphological clock, in its current form, cannot close the gap between the fossil record and molecular clock estimates for the date of origin of Placentalia. Our results suggest that morphological dating analyses as currently used tend to estimate ancient divergence estimates for clades when based on morphological data. This appears to be due to tip-dating approaches that lack sufficient constraints on ages prior to the root, and some morphological bias producing ancient age estimates. Morphological dating approaches cannot reconcile molecular estimates and a literal reading of the fossil record.

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DATA ARCHIVING

The doi for our data is doi:10.6084/m9.figshare.2570362.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Location of the node constraints on the node-dated phylogeny.
Figure S2. Absolute morphological rates through time, plotted at the mid-point of branches, following the protocol of Beck and Lee (2014).
Figure S3. Estimated ages of Placentalia from Cal3 (Bapst 2013) are younger than morphological clock approaches, but are dependent upon assumed sampling rates.
Table S1. Use of node constraints when additional node dating was employed alongside tip dating.
Table S2. Morphological clock ages indicate an ancient origin for Placentalia when using a uniform distribution on the root constraint
Table S3. Identical constrained, non-fixed topologies, results in ages that are much older than when node-calibration is used.
Table S4. Use of 13 internal node estimates, alongside the use of tip dating, results in node estimates that are younger than the use of tip dating alone.